The Inactivation of [Met⁵]-Enkephalin by Bestatin-Sensitive Aminopeptidase, Captopril-Sensitive Peptidyl Dipeptidase A and Thiorphan-Sensitive Endopeptidase-24.11 in Mouse Vas Deferens

Kazuko AOKI, Midori KAJIWARA and Tetsuo OKA*

Department of Pharmacology, School of Medicine, Tokai University,
Isehara 259-11, Japan

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Abstract—The enkephalin-hydrolyzing peptidases in mouse vas deferens were studied and compared with those in guinea pig ileum which had been characterized in the previous study. The present results showed that three distinct peptidases, bestatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A, and thiorphan-sensitive endopeptidase-24.11, played a critical role in the inactivation of enkephalin in mouse vas deferens, being consistent with the previous results obtained with guinea pig ileum. However, the data in both previous and present studies showed that the activity of the bestatin-sensitive aminopeptidase relative to that of either the captopril-sensitive peptidyl dipeptidase A or the thiorphan-sensitive endopeptidase-24.11 in guinea pig ileum was higher than that in mouse vas deferens, while the activity of either peptidyl dipeptidase A or endopeptidase-24.11 relative to that of aminopeptidase in mouse vas deferens was higher than that in guinea-pig ileum. In contrast to these three enzymes, both L-tyrosyl-L-tyrosine-sensitive dipeptidyl aminopeptidase and D-phenylalanine-sensitive carboxypeptidase were suggested not to be involved significantly in the inactivation of enkephalin in mouse vas deferens as well as guinea pig ileum.

Previous study has shown that three distinct enzymes, bestatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A (angiotensin I converting enzyme), and thiorphan-sensitive "enkephalinase" play a critical role in the inactivation of exogenously added enkephalins in the myenteric plexus-longitudinal muscle preparation of guinea pig ileum (1). Additionally, it has shown that these enzymes are likely to be located close to opioid receptors (1). On the other hand, both L-tyrosyl-L-tyrosine-sensitive dipeptidyl aminopeptidase and D-phenylalanine-sensitive carboxypeptidase have been indicated not to be involved significantly in the degradation of enkephalins in guinea pig ileum (1), although these two peptidases have been reported to be im-

plicated in the hydrolysis of enkephalin in the brain (2, 3). Since there are three opioid-receptor subtypes which are located on the adrenergic neurons in the mouse vas deferens while there are two opioid-receptor subtypes which exist on the cholinergic neurons in the guinea pig ileum, it is of interest to determine whether or not the enkephalin-hydrolyzing peptidases in the mouse vas deferens were the same as those in the guinea pig ileum.

Materials and Methods

Chemicals: Gifts of compounds which were gratefully received were bestatin from Nippon Kayaku (Tokyo, Japan), captopril and naloxone-HCl from Sankyo Company (Tokyo, Japan), phosphoramidon from Dr. T. Aoyagi, Institute of Microbial Chemistry (Tokyo, Japan), and thiorphan from Dr. M. Nozaki, Gifu University (Gifu, Japan). [Met⁵]-

* To whom all correspondence should be addressed.

Enkephalin and D-phenylalanine were purchased from Peptide Institute, Inc., (Minoh, Japan), and L-tyrosyl-L-tyrosine from Miles-Yeda, Ltd. (Israel).

In vitro isolated preparations: Male ICR-JCL mice weighing 30–40 g were used for this study. The vasa deferentia from mice were prepared and set up for electrical stimulation as described previously (4). The % inhibition of the stimulated muscle twitch produced by an opioid was plotted against the log concentration of the opioid to estimate the IC₅₀ (concentration of the drug to produce 50% inhibition of the twitch). When the effect of peptidase inhibitors on the enkephalin-induced depression of contractions was studied, they were given at least three minutes before the enkephalin administration. The % difference shown in the tables was calculated from % difference = [(IC₅₀ before each treatment – IC₅₀ after each treatment) / IC₅₀ before each treatment] × 100. The significance of % differences between IC₅₀ values of two adjacent groups shown in the table was determined by the paired Student's *t*-test.

Results

Enhanced effect of bestatin, captopril, thiorphan and phosphoramidon on [Met⁵]-enkephalin-induced inhibition: [Met⁵]-Enkephalin significantly inhibited the electrically-evoked contractions of mouse vas deferens. The inhibitory potency of [Met⁵]-enkephalin was augmented by the pretreatment of mouse vas deferens with bestatin (Table 1), an aminopeptidase inhibitor of bacterial origin (5). The enhanced effect of bestatin on the enkephalin-induced inhibition was dose-dependent and reached maximum at the dose of 100 μ M (Table 1).

Additionally, the inhibitory potency of [Met⁵]-enkephalin in bestatin-pretreated mouse vas deferens was dose-dependently enhanced by captopril (Table 2), an inhibitor of angiotensin I converting enzyme (6). The enhancement by captopril reached maximum at the dose of 1 μ M (Table 2).

Moreover, the inhibitory potency of [Met⁵]-enkephalin in bestatin-pretreated mouse vas deferens was enhanced by thiorphan (Table 3), an "enkephalinase" inhibitor (7). The

Table 1. Augmented effect of bestatin on the inhibitory potency of [Met⁵]-enkephalin in mouse vas deferens

Bestatin (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0	25.4 $\pm$ 5.8	1	
10	11.4 $\pm$ 2.6	2.16 $\pm$ 0.04	53.8 $\pm$ 0.95***
100	9.13 $\pm$ 2.2	2.82 $\pm$ 0.08	23.5 $\pm$ 1.4***
200	9.00 $\pm$ 2.1	2.85 $\pm$ 0.09	1.25 $\pm$ 0.9

Each value represents the mean $\pm$ S.E. of 4 experiments. ***P < 0.01.

Table 2. Enhanced effect of captopril on the inhibitory potency of [Met⁵]-enkephalin in the preparation pretreated with bestatin

Captopril (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0.1	6.26 $\pm$ 0.64	1	
1	3.84 $\pm$ 0.22	1.62 $\pm$ 0.09	37.5 $\pm$ 3.9***
10	3.87 $\pm$ 0.26	1.61 $\pm$ 0.07	0.5 $\pm$ 2.0

All preparations were pretreated with 100 μ M of bestatin immediately before the captopril administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P < 0.01.

augmentation by thiorphan was dose-dependent at the doses ranging from 0.1 to 10 μ M (Table 3). On the other hand, the potency of enkephalin in mouse was deferens pretreated with both bestatin and captopril was dose-dependently enhanced by thiorphan, reaching maximum at the dose of 1 μ M (Table 4). Similarly, phosphoramidon, an inhibitor of endopeptidase-24.11 (8), dose-dependently enhanced the inhibitory

potency of enkephalin in mouse was deferens pretreated with both bestatin and captopril (Table 5). The enhancement by phosphoramidon reached maximum at the dose of 1 μ M (Table 5). However, the IC₅₀ values of enkephalin in the preparation pretreated with bestatin, captopril and thiorphan were not significantly different from those pretreated with bestatin, captopril, thiorphan and phosphoramidon (Table 6), indicating that

Table 3. Enhanced effect of thiorphan on the inhibitory potency of [Met⁵]-enkephalin in the preparation pretreated with bestatin

Thiorphan (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0.1	6.46 $\pm$ 0.36	1	
1	2.69 $\pm$ 0.10	2.40 $\pm$ 0.07	58.0 $\pm$ 1.1***
10	1.73 $\pm$ 0.05	3.74 $\pm$ 0.12	35.8 $\pm$ 1.3***

All preparations were pretreated with 100 μ M of bestatin immediately before the thiorphan administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P<0.01.

Table 4. Enhanced effect of thiorphan on the inhibitory potency of [Met⁵]-enkephalin in the preparation pretreated with both bestatin and captopril

Thiorphan (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0	3.65 $\pm$ 0.32	1	
0.1	2.14 $\pm$ 0.31	1.75 $\pm$ 0.10	42.4 $\pm$ 3.4***
1	1.15 $\pm$ 0.20	3.34 $\pm$ 0.30	47.0 $\pm$ 2.4***
10	1.10 $\pm$ 0.16	3.40 $\pm$ 0.20	1.05 $\pm$ 3.8

All preparations were pretreated with both 100 μ M of bestatin and 1 μ M of captopril immediately before the thiorphan or vehicle administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P<0.01.

Table 5. Enhanced effect of phosphoramidon on the inhibitory potency of [Met⁵]-enkephalin in the preparation pretreated with both bestatin and captopril

Phosphoramidon (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0	3.22 $\pm$ 0.15	1	
0.1	1.72 $\pm$ 0.07	1.88 $\pm$ 0.03	46.8 $\pm$ 0.81***
1	1.27 $\pm$ 0.05	2.55 $\pm$ 0.15	25.5 $\pm$ 4.2***
10	1.33 $\pm$ 0.05	2.45 $\pm$ 0.18	4.5 $\pm$ 3.3

All preparations were pretreated with both 100 μ M of bestatin and 1 μ M of captopril immediately before the phosphoramidon or vehicle administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P<0.01.

Table 6. Negative effect of phosphoramidon (PH) on the inhibitory potency of [Met⁵]-enkephalin in the preparation pretreated with bestatin, captopril and thiorphan (TH)

TH (μ M)	PH (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0	0	3.53 $\pm$ 0.32	1	
1	0	1.00 $\pm$ 0.05	3.54 $\pm$ 0.28	71.2 $\pm$ 2.5***
1	1	1.06 $\pm$ 0.07	3.37 $\pm$ 0.31	5.58 $\pm$ 2.6

All preparations were pretreated with both 100 μ M of bestatin and 1 μ M of captopril immediately before the TH or vehicle administration. PH was given immediately after the TH administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P<0.01.

Table 7. Negative effects of D-phenylalanine (D-Phe) and L-tyrosyl-L-tyrosine (T-T) on the inhibitory potency of [Met⁵]-enkephalin in the preparation pretreated with bestatin, captopril and thiorphan

D-Phe (μ M)	T-T (μ M)	IC ₅₀ (nM)	Ratio of potency	% Difference
0	0	1.47 $\pm$ 0.19	1	
0	10	1.39 $\pm$ 0.16	1.05 $\pm$ 0.06	4.26 $\pm$ 5.3
0	0	1.10 $\pm$ 0.06	1	
100	0	1.02 $\pm$ 0.11	1.10 $\pm$ 0.09	6.73 $\pm$ 18

All preparations were pretreated with 100 μ M of bestatin, 1 μ M of captopril and 1 μ M of thiorphan immediately before the D-Phe, T-T or vehicle administration. Each value represents the mean $\pm$ S.E. of 8 experiments.

enkephalin-hydrolyzing peptidase inhibited by thiorphan was identical to that inhibited by phosphoramidon.

Negative effect of L-tyrosyl-L-tyrosine or D-phenylalanine on the magnitude of [Met⁵]-enkephalin-induced inhibition: In contrast to bestatin, thiorphan, captopril and phosphoramidon, neither L-tyrosyl-L-tyrosine, an inhibitor of amino-terminal-directed dipeptidylpeptidase (9), nor D-phenylalanine, a carboxypeptidase inhibitor (10), enhanced the inhibitory potency of [Met⁵]-enkephalin in the mouse vas deferens pretreated with bestatin, captopril and thiorphan (Table 7).

Inhibitory actions of peptidase inhibitors on contractions after the [Met⁵]-enkephalin administration: When peptidase inhibitors such as bestatin, captopril and thiorphan at the dose producing the maximum inhibition were added to the bath after the enkephalin administration, they produced the naloxone-reversible inhibition of contractions of mouse vas deferens (Fig. 1), although peptidase inhibitors by themselves did not produce the

1. [Met⁵]-Enkephalin, 10⁻⁹ M
2. Bestatin, 10⁻⁴ M
3. Captopril, 10⁻⁶ M
4. Thiorphan, 10⁻⁶ M
5. Naloxone, 10⁻⁶ M

Fig. 1. Naloxone-reversible inhibitory actions of peptidase inhibitors on electrically-evoked contractions of mouse vas deferens after [Met⁵]-enkephalin. Compounds were given to the bath at the dot.

inhibition of contractions when an opioid peptide was absent in the bath.

Discussion

All possible hydrolysis products of enkephalin have been reported to have very

weak or no agonist activity (11). Therefore, the fact that an inhibitor of a peptidase X, for example, significantly enhances the inhibitory potency of enkephalin indicates that peptidase X, when it is not inhibited, hydrolyzes the exogenously added enkephalin and decreases its inhibitory potency.

The fact that bestatin, an aminopeptidase inhibitor (5), significantly enhances the inhibitory potency of [Met⁵]-enkephalin indicates that the bestatin-sensitive aminopeptidase in mouse *vas deferens* hydrolyzes the exogenously administered enkephalin and decreases its inhibitory potency in mouse *vas deferens*. Additionally, the involvement of the captopril-sensitive peptidyl dipeptidase A in the hydrolysis of exogenously given enkephalin in mouse *vas deferens* is suggested by the fact that the inhibitory potency of [Met⁵]-enkephalin in bestatin-pretreated mouse *vas deferens* was significantly enhanced by captopril. Moreover, the involvement of the thiorphan-sensitive "enkephalinase" in the enkephalin hydrolysis is indicated by the fact that the potency of enkephalin in the mouse *vas deferens* pretreated with both bestatin and captopril was significantly augmented by thiorphan. Furthermore, the fact that the IC₅₀ value of [Met⁵]-enkephalin in the mouse *vas deferens* pretreated with both bestatin and captopril was significantly higher than that pretreated with bestatin, captopril and phosphoramidon suggests that the phosphoramidon-sensitive endopeptidase-24.11 in mouse *vas deferens* hydrolyzes the exogenously added enkephalin. Since phosphoramidon does not enhance the inhibitory potency of [Met⁵]-enkephalin in the mouse *vas deferens* pretreated with bestatin, captopril and thiorphan, the enzyme inhibited by thiorphan must be identical to that inhibited by phosphoramidon in mouse *vas deferens*, being consistent with the report of Matsas et al. (8) who have studied the hydrolysis of enkephalin with pig caudate synaptic membranes.

Since the magnitude of the enhancement of the inhibitory potency of [Met⁵]-enkephalin by thiorphan at the dose of 1 μ M was not significantly different from that at the dose of 10 μ M in the mouse *vas deferens* pretreated with both bestatin and captopril

while that at the dose of 1 μ M was significantly smaller than that at the dose of 10 μ M in the mouse *vas deferens* pretreated with bestatin alone, thiorphan at the dose of 10 μ M is likely to inhibit not only endopeptidase-24.11 but also peptidyl dipeptidase A, being consistent with the finding reported by Matsas et al. (12).

The enkephalin-hydrolyzing aminopeptidase is found to be inhibited maximally with bestatin at the dose of 100 μ M in the present study, since the enhanced effect of bestatin on the enkephalin-induced inhibition reaches maximum at the dose of 100 μ M. By the same analogy, the enkephalin-hydrolyzing endopeptidase-24.11 and peptidyl dipeptidase A are found to be inhibited maximally with either thiorphan or phosphoramidon and captopril at the dose of 1 or 1 and 1 μ M, respectively. The dose of each inhibitor to depress the activity of the enkephalin-hydrolyzing enzyme maximally in the mouse *vas deferens* obtained in the present study is the same as that previously obtained in the guinea-pig ileum (1).

In contrast to three peptidases such as aminopeptidase, peptidyl dipeptidase A and endopeptidase-24.11, both dipeptidyl aminopeptidase and carboxypeptidase are indicated not to be involved significantly in the degradation of exogenously added enkephalin in mouse *vas deferens*, since the IC₅₀ values of [Met⁵]-enkephalin in the mouse *vas deferens* pretreated with three peptidase inhibitors such as bestatin, captopril and thiorphan are not significantly different from those pretreated with three peptidase inhibitors plus either L-tyrosyl-L-tyrosine, which has been shown to be an inhibitor of an amino-terminal-directed dipeptidyl-peptidase (9), or D-phenylalanine, which has been reported to be a carboxypeptidase inhibitor (10).

The observation that bestatin, captopril and thiorphan inhibit the contractions of mouse *vas deferens* after the enkephalin administration and the inhibition is reversed by naloxone shows that the concentration of enkephalin in the vicinity of opioid receptors is significantly lower than that in the surrounding organ bath when these enkephalin-hydrolyzing peptidase inhibitors are absent.

In other words, peptidase inhibitors increase the concentration of enkephalin in the vicinity of opioid receptors by preventing the hydrolysis of enkephalin and therefore produce the naloxone-reversible inhibition of contractions. Since the exogenously given enkephalin can arrive in the vicinity of opioid receptors from various directions of the surrounding organ bath, the enkephalin-hydrolyzing peptidases in mouse *vas deferens* are likely to be located very close to opioid receptors, although the immunocytochemical study is apparently required to elucidate the precise topographical relation between opioid receptors and peptidases.

The data in the present study indicate that the bestatin-sensitive aminopeptidase, the captopril-sensitive peptidyl dipeptidase A, and both thiorphan and phosphoramidon sensitive endopeptidase-24.11 play a critical role in the inactivation of enkephalin in mouse *vas deferens*, being consistent with the previous data obtained in the guinea pig ileum (1). However, the data in both previous (1) and present studies show that the activity of the bestatin-sensitive aminopeptidase relative to that of either the captopril-sensitive peptidyl dipeptidase A or the thiorphan-sensitive endopeptidase-24.11 in guinea pig ileum is higher than that in mouse *vas deferens*, while the activity of either peptidyl dipeptidase A or endopeptidase-24.11 relative to that of aminopeptidase in mouse *vas deferens* is higher than that in guinea pig ileum. The significance of the difference existing between two preparations about the relative activity of three enkephalin-inactivating enzymes remains to be elucidated. Additionally, the significance of the presence of plural inactivating peptidases for one biologically active peptide also remains to be elucidated.

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